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Original article

Portal sinusoids' neutrophil plugging after experimental ischemia reperfusion liver injury

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ABSTRACT

The aim of the present study was the demonstration of a relationship between reduced liver microcirculation - after different periods of ischemia-reperfusion - and the number of neutrophils plugged to the microvasculature at reperfusion.

Ninety male Wistar rats were included in the study. These were subdivided into control group, 30min ischemia and 60min ischemia groups as well as 30min ischemia - 60min reperfusion and 60min ischemia - 60min reperfusion groups. Samples of liver tissue were obtained for the assessment of the number of neutrophils entrapped within portal sinusoids, after histochemical staining and morphometry. Hepatic microcirculation was estimated by laser-Doppler flowmetry. Additionally, ALT serum levels were evaluated, as an established marker of liver ischemia-reperfusion injury.

A course of 60min ischemia - 60min reperfusion resulted to a statistically significant increase of neutrophils plugged within liver sinusoids in relation to the 30min ischemia - 60min reperfusion [p=0.001]. Similarly, hepatic tissue microcirculation exhibited a flux recovery of 70% in 30min ischemia - 60min reperfusion group and only 57% in the 60min ischemia - 60min reperfusion group [p=0,001]. Serum ALT activities were found significantly increased during reperfusion, in both groups.

It is thus concluded that capillary perfusion failure occurring during liver reperfusion depends on ischemia time and is associated with a significant increase of neutrophils' accumulation into liver sinusoids.

Key words: liver, ischemia/reperfusion injury, neutrophils, microcirculation

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INTRODUCTION

Hepatic ischemia, and subsequent reperfusion, which occur during and after partial or total temporary interruption of liver blood flow, although unavoidable in distinct surgical maneuvers, such as extensive trauma or tumour resection, are associated with hepatic dysfunction.[1] Nowadays, the exact mechanisms of acute liver injury after ischemia-reperfusion are thought to involve a complex interaction of immediate cellular responses to Kupffer cell-derived cytokines and of subacute endothelial-leukocyte recruitment, which further facilitates liver injury.[2, 3, 4]

Although the clinical consequences of hepatic ischemia-reperfusion injury are well recognized, the exact mechanism that leads to the ultimate decline in liver function and eventual multisystem organ dysfunction remains elusive. Numerous studies dealing with free oxygen scavengers have suggested that reactive oxygen radicals may play a role.[2, 5, 6] Alternative hypotheses suggest that aberrant neutrophil and endothelial cell interactions may be the primary pathophysiologic mediator of liver damage.[7] Indeed, there is a growing body of evidence indicating that neutrophil adhesion to postcapillary venular endothelium is critical to the recruitment and accumulation of inflammatory phagocytes in postischemic tissues, where they act to exacerbate the cellular dysfunction induced by ischemia, both by

producing reactive radicals and by plugging the microvasculature, thus reducing nutritive flow.[8]

The estimation of microcirculatory blood flow and the quantitation of neutrophil plugged to the microvasculature at reperfusion, following different periods of ischemia, and the demonstration of a relationship between the time of ischemia, the reduction of blood flow at reperfusion - the no reflow phenomenon - and the number of plugged neutrophils, seem to be of crucial interest for the analysis of events that occur during liver ischemia and reperfusion.

Thus, the aim of the present study was to quantify accumulated neutrophils within hepatic sinusoids at reperfusion and match those measurements with those of liver microcirculation at different times periods of the ischemia.

MATERIAL AND METHOD

Animals

Male Wistar rats weighing 250-350gr were kept in a climatized environment with a 12h dark-light cycle, in accordance with the provisions of the Guide for the Care and Use of Laboratory Animals. All experimental procedures performed on these animals were reviewed and approved by the Department of Veterinary Medicine Guidelines on Animal Care and Use of the Ministry of Agriculture.

Experimental design

Five groups of animals were initially evaluated [n=14 per group]. Group 1, was the sham-operated group, not subjected to ischemia; groups 2 and 3 were the 30min and 60min ischemia groups, respectively; group 4 was the 30min ischemia and 60min reperfusion group and group 5 was the 60min ischemia and 60min reperfusion group. At the end of each study-period blood and liver tissue samples were obtained for laboratory tests and the respective group of rats was then sacrificed by ketamine overdose.

Another set of animals, forming only groups 4 and 5 [10 rats each one] was used for microcirculation measurements.

Study design

Twelve hours prior to the experiment the rats were deprived of food, but allowed free access to water. Anesthesia was main attained by the intramuscular injection of ketamine [50mg/kg] and xylazine [10mg/kg] and their body temperature was kept constant between 36.5°C and 37.5°C with a heating pad. Maintenance doses of anesthesia were injected as needed. A midline laparotomy was performed and the liver was mobilized by dividing the triangular and the falciform ligaments and all its peritoneal attachments. Baseline measurements of microcirculation as well as blood and liver samples from Group 1 were then obtained.

The liver hilus was exposed and the portal vein, hepatic artery and bile duct of the left lateral and median lobes were occluded by a small vascular clamp, distal to the origin of the vessels supplying the omental [caudate] and right lobes. The blood supply to those lobes thus remained uninterrupted, and portal blood flow was maintained through them without evidence of vascular congestion of the alimentary tract. This procedure is considered to yield about 70% partial ischemia of the liver by weight.

After 30min [Groups 2, 4] or 60min [Groups 3, 5] of normothermic ischemia, recirculation of blood through the ischemic lobes was achieved only in Groups 4 and 5 by simply removing the clamp. At the time of clamp removal, the second set microcirculation measurements as well as blood and liver samples were obtained from Groups 2 and 3. After 60min of reperfusion, the third set of microcirculation measurements was performed and samples were obtained from groups 4 and 5.

Serum marker of reperfusion injury

Blood samples obtained from the inferior vena cava for alanine aminotransferase [ALT] activity were stored in serum separator tubes and immediately centrifuged at 11.000rpm for 5min. A 10μl sample of serum was diluted in 0,9% saline and was analyzed using the serum multiple biochemical analyzer [Ektachem DTSCII, Johnson & Johnson, Rochester, NY] with appropriate standards.

Tissue neutrophil assessment

At the appropriate time, i.e. at the end of each experimentation period for each group, a sample of tissue from the left lateral lobe was taken and fixed in phosphate buffered formalin for further processing.

Formalin-fixed tissues were then paraffin embedded and 5μm thick sections were cut. Neutrophils [Ns] were stained, employing the naphthol AS-D chloroacetate esterase technique[9,10]. Naphthol AS-D chloroacetate was used as a substrate and new fuchsin as a coupler. Neutrophils were identified by positive staining and

morphology and were counted at 100X magnification using a Nikon Labophot microscope. The mean of Ns measured in 50 high-power fields was then calculated and the data was expressed as the number of Ns per high-power field [Ns/HPF].

Liver blood flow measurements

A single fiber probe was attached on the left lateral lobe by means of its self-adhesive latex sheet and remained attached throughout the whole study period, i.e. 30min ischemia plus 60min reperfusion or 60min ischemia plus 60min reperfusion, respectively. The probe was connected through the master-probe to the laser-Doppler apparatus.

The laser-Doppler flowmeter employed in the study was the Periflux PF2B [Perimed, Sweden]. The operating principle of this instrument has been described in detail elsewhere.[11] The self-adhesive single fibre probe [PF319:2L, Perimed, Sweden] used, is constituted of one optical fibre with an overall diameter of 0,5mm with a small latex sheet attached to its angular tip. This latex sheet adheres well to moist surfaces, and keeps the probe in position without glues or other mechanisms, thus permitting a very stable laser-Doppler signal to be obtained. All measurements were performed with a signal processing Periflux filter at 4kHz and time constant of the output amplifier at 0,2sec. The laser-Doppler flowmeter readings were continuously transferred and stored in a serially connected IBM-PS2 PC, by the use of the Perisoft software [Perimed, Sweden], for further analysis.

A 3-min measurement was performed just before blood flow occlusion, at the end of the period of ischemia and at the end of the reperfusion period. The individual preischemic reference value for each animal was set at 100% [i.e. normal erythrocyte flux] while the background signal obtained during ischemia was calibrated to be 0%. The resulting signals at various time points after onset of reperfusion thus could be reported as recovery of local flux in percentage of the individual reference.[12]

Statistical analysis

All values were expressed as the means ± the standard deviation of the mean [SD]. All parameters were then compared by means of one-factor ANOVA-test for repeated measurements. Differences were considered significant at the level of $p<0,05$.

RESULTS

We examine the effects of the total blood flow interruption to the median and left lateral hepatic lobes for either 30 min or 60 min with subsequent reperfusion for 1h. The fraction of the liver for which the vasculature was occluded represents 70% of the total liver mass.

Serum activities of ALT remained low during the ischemic period of 30 or 60min [32±10 versus 38±7, respectively]. A significant increase was seen only during reperfusion, the severity of the representing liver damage correlated well with the length of the ischemic period, i.e. a significant difference was found between 30min ischemia - 60min reperfusion and 60min ischemia - 60min reperfusion groups [102±12 versus 135±28, respectively, $p=0.01$].

As shown in Figures 1, 2, and 3, reperfusion after hepatic ischemia of either 30 or 60 min was associated with a significant recruitment of neutrophils into the liver. A 30min ischemia followed by 60min reperfusion resulted to a moderate neutrophils sequestration [209±35 Ns/HPF] as opposed to 60min ischemia followed by 60min reperfusion, which resulted to a statistically significant [$p=0.001$] increase of Ns number [263±55 Ns/HPF].

This ischemia-time depended neutrophils accumulation resulted to capillaries plugging and subsequently to a notable impairment of tissue perfusion. Thus, heparic tissue microcirculation measurements assessed against 0% flow during hepatic vessel occlusion revealed a local flux recovery of 70% after 30min ischemia - 60min reperfusion and of 57% after 60min ischemia - 60min reperfusion [$p=0.001$]. Figures 4 and 5.

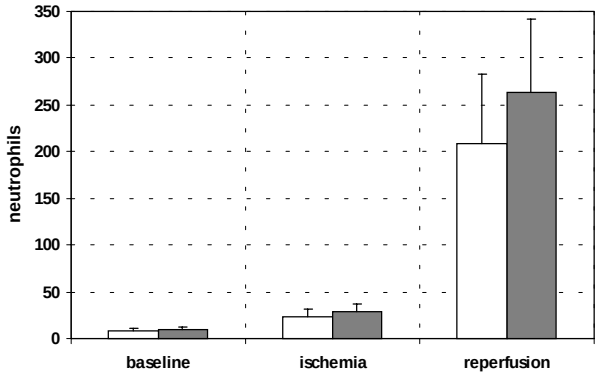

Figure 1. Neutrophil number [mean±SD] per study period. Open bars represent the 30min ischemia - 60min reperfusion groups and dark bars the 60min ischemia - 60min reperfusion groups.

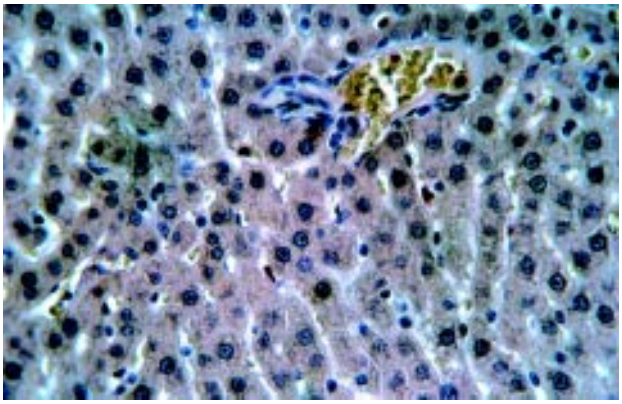

Figure 2. Rat liver section from a case with scant neutrophils after 30min ischemia - 60min reperfusion naphthol-ASD chloroacetate esterase stain X400

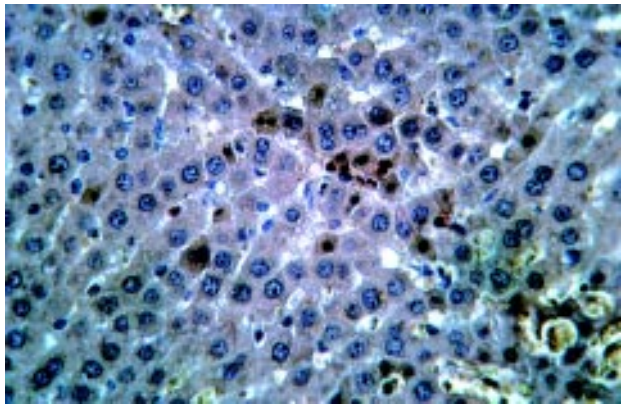

Figure 3. Rat liver section from a case with high number of neutrophils after 60min ischemia - 60min reperfusion naphthol-AS-D chloroacetate esterase stain X400

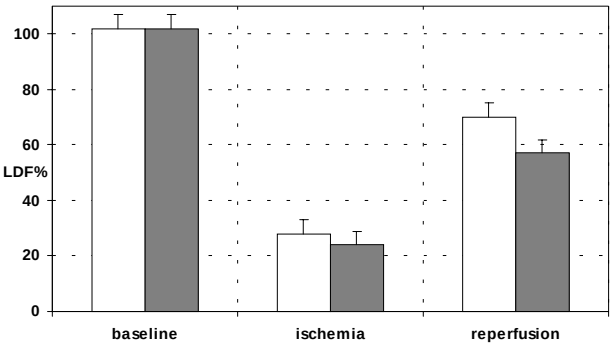

Figure 4. Liver microcirculation measurements [mean±SD] per study period. Open bars represent the 30min ischemia - 60min reperfusion groups and dark bars the 60min ischemia - 60min reperfusion groups

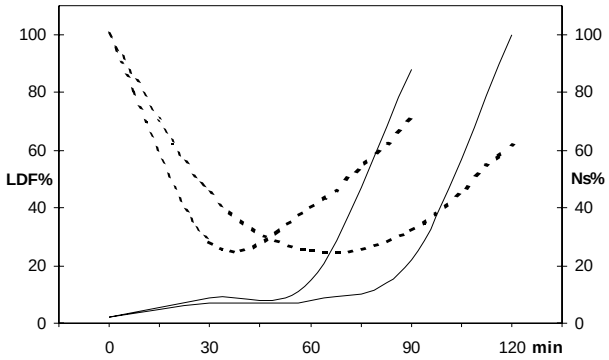

Figure 5. Correlation between liver microcirculation measurements [dot lines] and accumulated neutrophils [continues lines] after 30min ischemia - 60min reperfusion and after 60min ischemia - 60min reperfusion.

DISCUSSION

In this experimental study we provide evidence indicating a direct association between the number of neutrophils accumulated within the liver microvasculature and the elimination of hepatic microcirculation compared with pre-ischemia values, in relation to the ischemia time-lapse. For this purpose, the well-established model of 70% of the total liver mass ischemia was chosen, in order to avoid splanchnic congestion during ischemic period, which might interfere with the specific hepatic phenomena under investigation.[9, 12] Indirect proof that the experiments did not actually promote systemic alterations can be seen in the end-ischemic serum enzyme values of ALT, which remained quite normal until the free reflow establishment to the left and median liver lobes.

However, after a 60-min reperfusion period ALT exhibited a tremendous increase indicating parenchymal cell injury. According to the literature, periods of ischemia as brief as 30 minutes followed by reperfusion usually result in some degree of liver injury, the extent of which may be moderate and reversible or may be severe and extensive enough to cause cell death, organ failure, and ultimately host death. The causes that have been postulated to play a role in hepatic ischemia-reperfusion injury are considered to be free radical generation and attack of unsaturated lipids, proteins and nucleic acids; increased turnover of membrane phospolipids; disturbances of calcium homeostasis; Kupffer's cell-derived cytokines; and leukocyte-endothelial cell interactions.

In reference to the latter, our results demonstrate an ischemia time-dependent accumulation of neutrophils within the liver microvasculature. As it is assessed by tissue

morphometry, a 30 minutes ischemia followed by 60 minutes reperfusion resulted in a moderate neutrophils sequestration, as opposed to the 60 minutes ischemia followed by a similar 60 min reperfusion period, which lead to a statistically significant [p=0.01] increase in the number of neutrophils. Similarly, Jaeschke et al[9, 10] have demonstrated massive infiltration of leukocytes into the postischemic liver tissue associated with the development of hepatic injury, as indicated by increased ALT activities and hepatocellular necrosis, while the maximal number of leukocytes was found in the postischemic lobes during a 60min reperfusion after 60min of ischemia. Despite these data and the finding that continuous neutropenia induced by an anti-neutrophil monoclonal antibody drastically reduced the number of neutrophils infiltrating the liver and protected against reperfusion injury[10], they consider than oxidative stress and hepatocellular injury did not correlate with the number of neutrophils in the liver during this early reperfusion period.[13]

However, in accordance with previous work,[14] a very high value of purine nucleoside phosporilase was found in the serum after reperfusion. Since this is an enzyme predominantly localized in the endothelial cells, the hepatocyte is rather void of it, it can be deduced that pronounced alterations occur to the vascular endothelium upon reperfusion; such an endothelial damage is considered to be not only the matrix for passive neutrophil rolling and adherence but mainly the stimulator of adherence. Thus, it is now well established that during reperfusion, overproduction of toxic oxygen radicals and proteases as well as endothelial cell damage results in leucocyte adhesion and can cause tissue injury through capillary plugging, since it considerably extends the ischemic period of the liver. [15, 16, 17]

This event of capillary plugging appears to be the microvascular origin of the no-reflow phenomenon, as observed in whole organs during ischemia. In the absence of neutrophils the blood yield stress does not exert a sufficient resistance to cause obstruction of the capillaries, which otherwise have an unobstructed lumen, as seen by intravital microscopy.[17] Previous studies have demonstrated that leucocytes adhere to endothelial cells of both sinusoids and post-sinusoidal venules, but the present situation is that adherence of leucocytes to the hepatic venule did not completely occlude blood flow, but undoubtedly reduced the effective lumen of the venule and enhanced the resistance to flow. Thus, Koo,[18] reports a reduction in leukocyte velocity of approximately 80% of the corresponding erythrocyte velocity in the liver sinusoid during the reperfusion phase following a -30min ischemia.

Ischemia- reperfusion is characterized not only by the massive neutrophil infiltration into the post-ischemic tissue but by a failure of nutritive perfusion, is clearly demonstrated in the present work. By the use of laser-Doppler flowmetry, recognized as a reliable method for estimation of liver microcirculation and exhibiting a high sensitivity in perfusion alterations, we demonstrated a good correlation between the length of the ischemic interval -30 or 60 minutes- and the reduction in microcirculation. Thus the 30% reduction of capillary flow assessed after 30min ischemia - 60min reperfusion, increased to a percentage of 43% after 60min ischemia/ 60min reperfusion. Similarly, in such an ischemic model, hepatic blood flow during 30 to 120min of reperfusion is reported to be 30% to 75% of initial values[19] while Clemens et al[20] demonstrated that the number of reperfused sinusoids on the surface of the liver was reduced after ischemia and reflow. These findings are also consistent with the observation of Goto et al[19], that blood oxygenation index, assessed by reflectance spectrophotometry, remained low after long ischemia. Decrease in O_2 demand and O_2 supply might occur in the early stage of reperfusion to the extent that insufficient O_2 supply may be the dominant factor contributing to the decreased blood oxygenation index, which in turn leads to regional liver hypoxia. In addition, the decreased volume of the regional hepatic tissue hemoglobin indicates a decrease in vascular space or regional blood volume in the liver, suggesting that the decreased oxygen saturation index is due to the decreased blood flow associated with insufficient O_2 supply.[19]

We, therefore, acknowledging the role of plugged neutrophils in reducing microcirculation, tried to correlate the number of accumulate neutrophils with the ischemia-reperfusion-induced microcirculatory disturbances i.e. the no-reflow phenomenon in respect to the ischemia-time.

Thus a 30min ischemia followed by 60min reperfusion led to a 26-fold increase in the number of neutrophils and a 30% of perfusion reduction, while a 60min of ischemia followed by 60min reperfusion led to a 32-fold increase in accumulated neutrophils and a 43% reduction of flow [Figure 3]. Similarly, Komatsu et al[21], working in a rat model of only 25min ischemia followed by 30min of reperfusion, reported a 3.1-fold increase [from 18±7 to 57±4 cells/mm^2] in the number of neutrophils in the liver tissue, which is proportional to the 4.4-fold increase in myeloperoxidase activity. In addition, they stated that the cumulative number of neutrophil adherence to the

endothelium increased in parallel with the microcirculatory stasis of blood flow.

In conclusion, the present study led us to suggest that capillary perfusion failure occurring during reperfusion depends on ischemia time and is associated with a significant increase in neutrophils accumulation and plugging of sinusoids. As a consequence, sinusoidal plugging leads to impaired microcirculation and thus to compromised hepatocellular integrity and parenchymal function, a situation a major contribution of postischemic sinusoidal no-reflow to the manifestation of liver injury, by means of prolongation of hypoxic conditions during reperfusion.

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